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Duplex PCR for specific detection of *Escherichia coli* and its differentiation from other Enterobacteriaceae

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ABSTRACT

Escherichia coli is a normal inhabitant of gastrointestinal tract of humans and animals and one of the most important causes of bovine mastitis. Definitive identification of *E. coli* from other members of Enterobacteriaceae remains ambiguous. The present study describes a duplex PCR, targeting 2 housekeeping genes, the *lacy* (lactose permease) and *pho*A (alkaline phosphatase) for the reliable detection of *E. coli* that enables its differentiation from biochemically and phylogenetically related bacteria. The assay was evaluated employing 19 ATCC (American type culture collection) reference strains of Enterobacteriaceae family. Validation of the assay with *E. coli* (154) isolated from milk and faeces rendered the assay to be specific. The results suggest that the technique can be used for accurate detection of *E. coli* and thus can be adapted for testing bacteriological safety of milk, for field applications, and in laboratories handling clinical samples. This PCR (polymerase chain reaction) can successfully distinguish *E. coli* including *E. coli* O157 from *Shigella* spp and other related enterobacteria, emphasizing its relevance and utility in studies related to *E. coli* infection.

Key words: Duplex polymerase chain reaction, Enterobacteriaceae, *Escherichia coli*

E. coli is associated with a wide range of clinical conditions in animals including mastitis, calf scours, enterotoxicity etc. Its importance as an indicator organism in testing the microbiological quality of foods and water cannot be over emphasized. Hence, it has assumed importance in all public health investigations regarding animal products meant for export (Wenz *et al.* 2006, WHO 2011,Gautam *et al.* 2012). Isolation and identification of *E.coli* by culture is a robust and widely used method, albeit significantly low in specificity being one of its several limitations.

Moreover, as reported earlier it is practically impossible to distinguish *E.coli* and *Shigella* on the basis of their DNA sequences. Hence, *Shigella* strains were considered as pathovars of *E.coli* based on the similarity among the housekeeping and plasmid genes shared between strains of both species. Most of the earlier reports either involving *E.coli* specific PCR or 16Sr RNA for rapid detection of *E.coli* were found to lack the specificity since none of the assays could rule out the presence of *Shigella* sp. (Pavlovic *et al.* 2011, Gautam *et al.*2012).

Therefore, the detection of *Shigella* and simultaneous differentiation from all other Enterobacteriaceae was necessary to be addressed in analytical evaluation of every *E. coli*-specific PCR assay.

Herein, we describe a simplified duplex PCR method targeting *lac*Y and *pho*A genes which allows specific identification of *E. coli* differentiating it from all other species of Enterobacteriaceae.

MATERIALS AND METHODS

Bacterial strains

E. coli strains (158) comprising 99 isolated from bovine milk including *E. coli* O157, 55 isolated from faecal samples of poultry and cattle, and 4 ATCC (American type culture collection) strains were used. Non *E. coli* ATCC reference strains, *Shigella sonnei, Shigella flexneri, Shigella boydii, Salmonella enterica, Klebsiella pneumonia, Pseudomonas aeruginosa, Citrobacter koseri, Hafnia alvei, Ewingella americana, Serratia marcescens, Edwardsiella tarda, Proteus mirabilis, Enterobacter aerogenes, Erwinia amylovora* and *Pasteurella multocida* and *Staphylococcus aureus* were also included.

Primers

With the recent updated public database, primers specific for the *lac*Y (lactose permease) and *pho*A (bacterial alkaline phosphatase) genes were designed using primer-3 software

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Table 1. Oligonucleotide primers

Gene	Accession number	Primer designation	Oligonucleotide sequence (5'-3')	Amplicon (bp)	Annealing temperature (60 ºC)
*lac*Y	NC_000913	LACy	CTACCGGTGAACAGGGTACGGTCGCTGAAAA ACGCACTTC	289	60 ºC
*pho*A	FJ546461	PHOa	GGTAACGTTTCTACCGCAGAGTTGCAGGGTTGGTAC ACTGTCATTACG	468	60 ºC

lacy, Lactose permease; *pho*A, bacterial alkaline phosphatase.

(http://frodo.wi.mit.edu/primer3/ (Rozen and Skaletsky 2000) and custom synthesized. The primer sequences, the annealing temperatures and the length of the amplified products are summarized in Table 1. The reported primers based on 16SrRNA (16E1+16E2+16E3) (Gautam *et al.* 2012) were synthesized and evaluated for its specificity using the various control strains included in the current study by PCR as described by Gautam *et al. (*2012).

DNA extraction and duplex PCR amplification

Total bacterial genomic DNA was extracted from overnight grown *E. coli* reference strains in nutrient broth using DNA mini kit and concentration was determined by another kit. In the similar way, DNA extracted by boiling method also worked equally well, reducing the cost and time.

The reaction was performed in a volume of 25 µl with primer concentration of 0.15µM *lac*Y*,* and 0.6µM *pho*A and 100 ng template DNA in 1 × PCR buffer containing 10 mM Tris-HCl, pH 8.8, 2 mM $MgCl_2$, 50 mM KCl, 1.5 U of *Taq* DNA polymerase, 200 mM dNTPs. The cycling conditions include, initial denaturation at 94 ºC for 5 min followed by 30 cycles with denaturation at 94 ºC for 30 sec, annealing at 60ºC for 30 sec and extension at 72 ºC for 45 sec. The amplified products were analyzed onto 1.5% agarose gel containing ethidium bromide (10 µg ml^{-1}). The analysis of specificity was done with the 15 non *E. coli* reference strain. To validate the assay, a total of 99 isolates of *E. coli* from bovine milk and 55 faecal *E. coli* isolates from poultry and cattle were subjected to duplex PCR.

Detection limit of duplex PCR

To determine the detection limit of DNA, the concentration of purified DNA extracted from target bacterial species were determined using nanodrop and 10–fold serial dilutions of purified DNA were made and subjected to duplex PCR.

RESULTS AND DISCUSSION

Use of PCR based identification methods in identification of bacteria has increased the sensitivity and reliability associated with the technique, in comparison to the conventional culture methods. However, with regard to *E. coli,* its specific identification and differentiation from *Shigella* spp. by targeting single phylogenetically conserved region of the genome is challenging due to the high similarity of the conserved sequences with other members of *Enterobacteriaceae* family across the genome (Pavlovic *et al.* 2011). The close relationship between *E. coli* and *Shigella* species hampers their differentiation. Therefore, it is obvious that many *E. coli* could be misidentified as *Shigella* and vice-versa (Horakova *et al.*2008). In this study, a duplex PCR assay was developed which enabled accurate identification of *E. coli*, simultaneously differentiating from *Shigella* spp and other Enterobacteriaceae members targeting two housekeeping genes namely, the *lac*Y coding for lactose permease and *pho*A coding for bacterial alkaline phosphatase. *E. coli* shares about 95–97% similarity in genome with certain species of *Shigella* and varies in only about 3 base pairs in the 16S region due to a close evolutionary relationship (Chakravorty *et al.*2007). Due to the high similarity of the conserved sequences with other members of Enterobacteriaceae family across the genome, it imposes difficulty in differentiating *E. coli* and *Shigella* species from each other as well from other members of Enterobacteriaceae.

Gautam *et al.* (2012) recently reported a PCR based detection of *E. coli* in milk by targeting 16S rRNA gene which when employed in the current study gave an expected 584 bp PCR product corresponding to *E. coli* as well with *Shigella* spp. Thus, the evaluation of specificity of the reported primers showed amplification with *Shigella* spp. rendering the assay non specific (Fig. 1).

Previous PCR methods based on housekeeping genes had its shortcomings because housekeeping genes are not only present in *E. coli* but also in other closely related genus

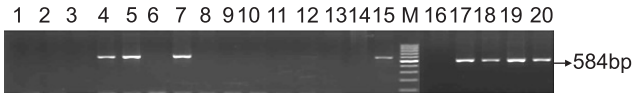


Fig. 1. Evaluation of specificity of PCR amplification with various control strains using reported primers16E1+16E2+16E3 (Gautam *et al.*2012). Lane 1: *Salmonella* ATCC 13428; lane 2: *Enterobacter* ATCC 13048; lane 3: *Pseudomonas* ATCC 7700; lane 4: *Shigella flexneri* MTCC 1457; lane 5: *Shigella boydii* ATCC 8700; lane 6: *Klebsiella pneumoniae* ATCC 4209; lane 7: *Shigella sonnei* ATCC 25931*;* lane 8: *Citrobacter* ATCC 25408, Lane 9: *Hafnia* ATCC 23280; lane 10: *Serratia* ATCC 4002; lane 11: *Edwardsiella* ATCC *23672;* lane 12: *Erwinia* ATCC 7400; lane 13: *Pasteurella* ATCC 10544; lane 14: *Staphylococcus aureus*; Lane 15: Positive control; lane M: 100 bp ladder; lane 16: No template control; lanes 17–20: *E. coli* ATCC (25922, 35218, 700336, 8739).

of the *Enterobacteriaceae* family and thus hindered designing of a specific primer for *E. coli* (Sandhya *et al.* 2008). As a result, multiplex PCR approach targeting more than one set of housekeeping genes was designed. Various combinations of genes namely, the *lac*Z, *uid*A, *rpo*S, *mal*B, *alr, rrn*B and others were used (Maheux *et al.* 2009) but the problem of differentiating *E. coli* and *Shigella* spp. still persisted. The mPCR described by Horakova *et al.* (2008) though seemed to work well, resulted in non-specific amplification on being applied to additional *Shigella* strains (Pavlovic *et al.* 2011). Horakova *et al.* (2008), has developed a mPCR using 4 targets and reported the appearance of 4 bands corresponding to the primers to be *E. coli* and 3 or less bands to be non *E. coli*. However, the duplex PCR developed in the present study simplified the mPCR method (Horakova *et al.*2008) by designing two primers targeting *pho*A and *lac*Y which allowed successful differentiation of *E. coli* from all other members of *Enterobacteriaceae* including *Shigella* species.

Evaluation of accuracy of duplex PCR with *E. coli* and other enterobacteriaceae members showed two fragments corresponding to the *lac*Y (289bp) and *pho*A (468bp) only for *E. coli* (Fig.2). Whereas, one or no amplicon was observed for non- *E. coli* strains including *Shigella* spp. namely *S. sonnei, S. flexneri* and *S. boydii* (Table 2).

The threshold limits of the detection assay, where the specific two DNA fragments could be amplified, was 1ng of bacterial DNA (Fig. 3).

Further, as *E. coli* is often isolated from udders of animals suffering from severe clinical mastitis, the duplex assay was evaluated with *E. coli* strains isolated from milk samples (Griesbeck-Zilch 2008). Isolates (154) of *E. coli* from bovine milk (99) and faecal samples of poultry and cattle (55) were subjected to duplex PCR. All the isolates

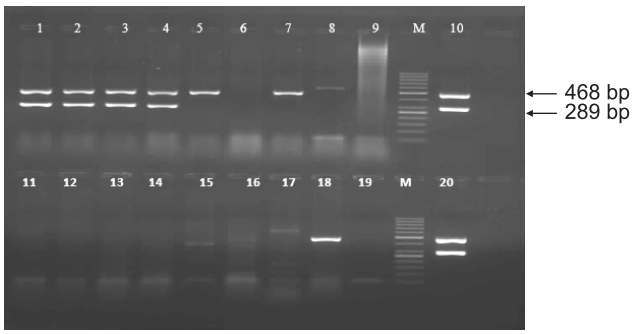


Fig. 2. Duplex PCR amplification of DNA from various control strains using *lac*Y (289 bp) and *pho*A (468 bp). Lanes 1–4: *E. coli* ATCC (25922, 35218, 700336, 8739); lane 5: *Shigella flexneri* MTCC 1457; lane 6: *Salmonella* ATCC 13428; lane 7: *Shigella sonnei;* lane 8: *Klebsiella pneumoniae* ATCC 4209; lane 9: *Pseudomonas* ATCC 7700; lane M: 50 bp ladder; lane 10: Positive control; lane 11: *Citrobacter* ATCC 25408; lane 12: *Hafnia* ATCC 23280; lane 13: *Serratia* ATCC 4002; lane 14: *Edwardsiella* ATCC *23672;* lane 15: *Enterobacter* ATCC 13048; lane 16: *Erwinia* ATCC 7400; lane 17: *Pasteurella* ATCC 10544; lane 18: *Shigella boydii* ATCC 8700; lane 19: no template control; lane M: 50 bp ladder; lane 20: positive control.

Table 2. Evaluation results of duplex PCR assay

Ref Strain	Bacteria	*lac*Y	*pho*A
A 25922	*Escherichia coli*	+	+
A 35218	*Escherichia coli*	+	+
A 700336	*Escherichia coli*	+	+
A 8739	*Escherichia coli*	+	+
A 25931	*Shigella sonnei*	-	+
M 1457	*Shigella flexneri*	-	+
A 8700	*Shigella boydii*	-	+
A13428	*Salmonella* Enterica	-	-
A 4209	*Klebsiella pneumonia*	-	-
A 7700	*Pseudomonas aeruginosa*	-	-
A 25408	*Citrobacter koseri*	-	-
A 23280	*Hafnia alvei*	-	-
A 33850	*Ewingella Americana*	-	-
A 4002	*Serratia marcescens*	-	-
A 23672	*Edwardsiella tarda*	-	-
A 4630	*Proteus mirabilis*	-	-
A 13048	*Enterobacter aerogenes*	-	-
A 7400	*Erwinia amylovora*	-	-
A 10544	*Pasteurella multocida*	-	-

showed amplification of 2 fragments derived from the *lac*Y, and *pho*A genes. Thus, a clear differentiation of *E. coli* strains from species with similar biochemical and genetic properties, such as *Shigella* spp was attained (Fig. 4) confirming its accuracy and reliability for detection of *E. coli* from diverse samples. The PCR assay developed was also validated on both O157 and non-O157 *E. coli* strains.

In conclusion, our duplex approach seems to be an adequate tool for reliable identification of *E. coli*. This will be of the highest importance for testing bacteriological safety of milk, when DNA is extracted directly from milk by using protocol reported by Cremonesi *et al.* (2006). The enhanced specificity of the method makes it a highly reliable and useful method for accurate differentiation from *Shigella* sp. and from other Enterobacteriaceae thus emphasizing

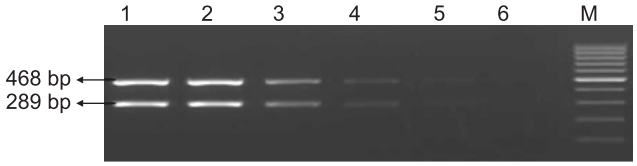


Fig. 3. Sensitivity of duplex PCR assay after amplification with template DNA from *E. coli* ATCC 25922 using primers *lac*Y and *pho*A. Lanes 1–6: 1000ng, 100 ng, 10 ng, 1ng, 0.1ng, 0.01ng; and M: 100bp ladder.

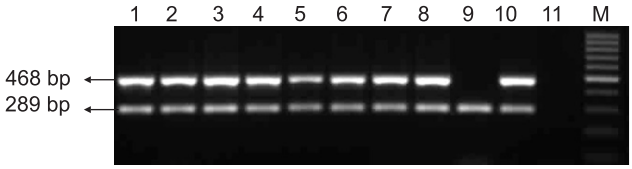


Fig. 4. Duplex PCR amplification of DNA from *E. coli* isolated from diverse samples using *lac*Y (289 bp) and *pho*A (468 bp). Lanes 1–8: *E. coli* isolates; lane 9: negative control, lane 10: Positive control; lane 11: no template control; lane M: 100 bp ladder.

its relevance and utility in studies related to *E. coli* infection.

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